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ROVISIONAL APPLICATION FOR PATENT COVER SHEET

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This is a request for filing a Provisional Application for Patent under 37 CFR 1.53(c)

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Date of Deposit: June 12, 2002
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For: **P90 RSK INHIBITORS AS THERAPEUTICS AND INVESTIGATIVE TOOLS**

4 Sheets of specification.
3 Sheets of drawings.

University of Virginia Patent Foundation claims small entity status as a nonprofit organization (37 CFR §1.9(e) and §1.27(d)). Therefore, please charge the Small Entity Fee of $80 to **Deposit Account No. 50-0423.**

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This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The government has certain rights in the invention.

YES ☐ NO ☒ Grant No. ________

Dated: June 12, 2002

Respectfully submitted,

By: ____________________
John P. Breen (Reg. No. 38,833)

p90 RSK Inhibitors as Therapeutics and Investigative Tools

Background

Improper regulation of the Mitogen-activated Protein Kinase (MAPK) pathway is a distinguishing characteristic in many tumors as well as neurological diseased states such as epilepsy. p90 Ribosomal S6 Kinase (RSK) is a downstream component of the MAPK signaling pathway and is activated by MAPK. Therefore, unregulated stimulation of the MAPK pathway results in unregulated RSK catalytic activity. The contribution of upstream components such as Epidermal Growth Factor Receptor (EGFR) and the products of the proto-oncogenes c-src, ras, and raf to diseased states has been well documented. Activation of any of these signaling molecules results in physiological responses by the cell. However, the extent to which these physiological responses function through RSK is unknown. Until now, it has been difficult to define the role that RSK plays in normal and diseased states because a specific inhibitor of RSK catalytic activity had not been identified. A RSK specific inhibitor will be useful as a tool for investigating RSK function under normal conditions and under diseased conditions in which regulation of the MAPK signaling pathway has been compromised.

Brief Summary of the Invention

A methanolic extract from the plant *Forsteronia refracta* has been discovered to contain several potent RSK inhibitors. The compounds have been shown to be specific inhibitors of RSK *in vitro* and *in situ*. In addition, inhibition of RSK by the compounds halts proliferation of Ha-ras-transformed NIH/3T3 cells while having little effect on the proliferation rate of parental NIH/3T3 cells. Therefore, the present invention identifies RSK as a target for therapeutic intervention in diseased states in which the disease or the symptoms can be ameliorated by inhibition of RSK catalytic activity or in combination with additional therapies. Any compound for which direct inhibition of RSK constitutes all or part of its physiological effect can fulfill this objective. This includes, but is not limited to the compounds or analogs thereof from the plant extract described herein.

Brief Summary of the Drawings

FIGURE 1: Molecular structure of SL0101-1, SL0101-2 and SL0101-3

FIGURE 2: Inhibitory potency of SL0101-1, SL0101-2 and SL0101-3. The catalytic activity of RSK in the presence of increasing concentrations of each compound was measured. The IC_{50}s of each compound was determined.

FIGURE 3: *In vitro* specificity of SL0101. The influence of SL0101 on the catalytic activity of several protein kinases was examined.

FIGURE 4: In situ examination of SL0101 efficacy. SL0101 reduced phosphorylation of the in vivo RSK substrates Estrogen Receptor a (ER a) and pp140. Phosphorylation of ER a and pp140 was detected with anti-phospho-specific antibody recognizing the RSK phosphorylation site in these substrates. Presence of the inhibitor did not alter phosphorylation of RSK as indicated by the reduced mobility of RSK during SDS-PAGE. SL0101 did not inhibit phosphorylation of MAPK as indicated by the anti-phospho-specific antibody recognizing phosphorylated, active MAPK.

FIGURE 5: SL0101 inhibits proliferation of transformed cells but not parental cells. Inhibition of RSK by SL0101 halts proliferation of Ha-ras-transformed NIH/3T3 cells but has little effect on the proliferation rate of non-transformed NIH/3T3 cells compared to that observed with vehicle alone. Proliferation was measured using Promega CellTiter-Glo™ Luminescent cell viability assay.

Detailed Description of the Invention

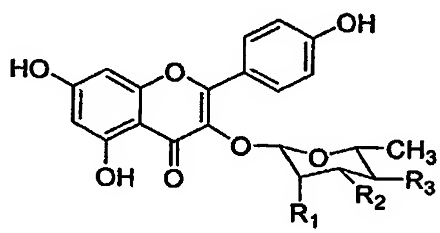
p90 Ribosomal S6 Kinase (RSK) is a serine/threonine kinase that is a downstream component of the Mitogen-activated Protein Kinase (MAPK) signaling pathway and is activated by MAPK. MAPK is activated by several diverse signals under normal conditions and is hyperactive in many diseased states due to improper regulation of the signaling pathway. Activation of MAPK results in increased RSK catalytic activity. However, defining the physiological function of RSK activation under normal and diseased conditions has been difficult because there are no RSK-specific inhibitors.

We have identified potent and specific RSK inhibitors in methanolic extracts from the plant *Forsteronia refracta*. Purification and structure determination of three inhibitors have been completed. The methanolic extract from wood stem and stem bark of *Forsteronia refracta* was applied to a polyamide 6S column, which was washed successively with H_2O, 1:1 H_2O- MeOH, 9:1 CH_2Cl_2-MeOH, 1:1 CH_2Cl_2-MeOH and 9:1 MeOH-NH_4OH to afford five fractions. The 9:1 CH_2Cl_2-MeOH and 1:1 CH_2Cl_2-MeOH fractions showed stronger inhibition of RSK than starting material. The 1:1 CH_2Cl_2-MeOH fraction was further fractionated on a diol gel column. The column was eluted successively with CH_2Cl_2, 99:1 CH_2Cl_2-MeOH, 95:5 CH_2Cl_2-MeOH, 90:10 CH_2Cl_2-MeOH and MeOH to give five fractions. Among these, the 95:5 CH_2Cl_2-MeOH, 90:10 CH_2Cl_2-MeOH and MeOH fractions showed the same or stronger activity than the starting material. The 95:5 CH_2Cl_2-MeOH fraction was fractionated repeatedly on a C_{18} reverse phase HPLC column (250'10 mm); elution was carried out with 65:35 MeOH-H_2O and UV detection was at 265 nm. Two compounds, SL0101-1 and SL0101-2 were obtained as amorphous pale yellow powders. On the basis of its ^1H NMR spectrum and positive APCI-MS, SL0101-1 was found to be kaempferol 3-a-L-(3",4"-diacetyl) rhamnopyranoside (**2**)[1] (figure 1). SL0101-2 was proved to be kaempferol 3-a-L- (2",4"-diacetyl) rhamnopyranoside (**1**)[2].

The 90:10 CH_2Cl_2-MeOH fraction from above diol column was also fractionated repeatedly on a C_{18} reverse phase HPLC column using 45:55 H_2O-MeOH as the eluant and UV detection at

275 nm. The active constituent, SL0101-3, was obtained as an amorphous powder. On the basis of its ^1H NMR and ^{13}C NMR data, the compound was found to be kaempferol-3-a-L- (4"-acetyl) rhamnopyranoside (3)[3].

Although all these compounds have been reported previously, there is no report on the biological activity of the acetyl kaempferol-3-a-L-rhamnopyranosides. The isolation of other active principles is still in progress. In accordance with one embodiment of the present invention a RSK-specific inhibitory composition is provided. The composition comprises a compound represented by the general structure



wherein R_1, R_2 and R_3 are independently selected from the group consisting of H, hydroxy -$OCOR_4$ and C_1-C_4 alkoxy, and R_4 is H or C_1-C_4 alkyl. In one embodiment R_1 and R_2 are independently selected from the group consisting of hydroxy and -OAc and R_3 is -OAc. The compositions may further comprise a pharmaceutically acceptable carrier.

The individual compounds, SL0101-1, SL0101-2 and SL0101-3 (see figure 1), are collectively referred to as SL0101. SL0101-1, SL0101-2 and SL0101-3 are ATP-mimetics (data not shown) that inhibit RSK *in vitro* with IC_{50}s of 90 nM, 580 nM and 190 nM, respectively (figure 2). However, they do not inhibit the evolutionarily related p70 S6 kinase and Mitogen- and Stress-activated Protein Kinase (MSK). In addition, they do not inhibit the prototypical serine/threonine kinase Protein Kinase A or the tyrosine kinase Focal Adhesion Kinase (FAK) (figure 3).

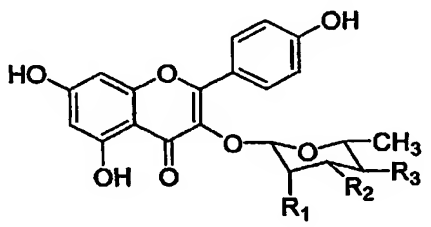
Specific inhibition of RSK *in situ* was determined by incubation of MCF-7 cells in the presence or absence of increasing concentrations of extract fraction enriched in SL0101 prior to stimulation of the MAPK pathway with phorbol dibutyrate (PDB). The presence of the RSK inhibitor eliminated phosphorylation of the RSK substrates Estrogen Receptor alpha (ERa) and pp140 as determined using phospho-specific antibodies developed using the RSK phosphorylation site in the ERa as the antigen (figure 4). However, the inhibitor did not alter phosphorylation of RSK by MAPK as indicated by the generation of RSK with reduced mobility observed following SDS-PAGE. The inhibitor did not influence activation of MAPK by the MAPK Kinase, MEK as determined by the phospho-specific antibody recognizing active MAPK. Therefore, SL0101 did not inhibit the catalytic activity of Protein Kinase C (PKC), RAF, MEK, or MAPK because these kinases are essential to cause phosphorylation of RSK in cells

stimulated with PDB. Thus, SL0101 is a RSK-specific inhibitor *in situ* as well as *in vitro* and can be used as an investigative tool for defining the function of RSK *in situ.*

We have determined that inhibition of RSK eliminates proliferation of Ha-ras-transformed NIH/3T3 cells without influencing the proliferation rate of non-transformed NIH/3T3 cells. Ha-ras-transformed NIH/3T3 cells or parental NIH/3T3 cells were incubated in the presence of vehicle, 50 mM SL0101, or 50 mM PD 98059, a MEK-specific inhibitor. The presence of SL0101 eliminated Ha-ras-transformed NIH/3T3 cell proliferation over a 48 hour time course, even in the presence of 10 % fetal calf serum (figure 5). However, SL0101 had little influence on the rate of parental NIH/3T3 proliferation compared to that observed in the presence of vehicle. An influence on the proliferation rate by the MEK inhibitor, PD 98059 was observed only when cells were incubated in the presence of low concentrations of fetal calf serum (0.1 - 1 %) (data not shown).

Thus, RSK-specific inhibitors have been shown to abolish proliferation of a transformed cell without substantially altering the rate of non-transformed cell growth. Therefore, SL0101 is not toxic to non-transformed cells. These data suggest that RSK-specific inhibitors can be used as an anti-cancer therapy by abolishing the growth of malignant tumors without toxic effects on the normal tissues. In addition, because SL0101 inhibits RSK specifically *in situ* (figure 4) without toxic effects, RSK inhibitors can be also be used as therapeutic interventions in non-terminal diseased states such as epilepsy in which the MAPK signaling pathway is improperly regulated. In accordance with one embodiment, the RSK-specific inhibitors of the present invention are used to treat cancer and neurological disorders. "Treating" as used herein includes administering therapy to prevent or cure the disease (for example, for cancer this includes inhibiting tumor initiation and progression), as well as alleviating the symptoms associated with the disease/disorder.

D. DRAWINGS OR SUPPORT MATERIAL



	R_1	R_2	R_3	IC_{50}
SL0101-1 (2)	OH	OAc	OAc	90 nM
SL0101-2 (1)	OAc	OH	OAc	580 nM
SL0101-3 (3)	OH	OH	OAc	190nM

FIGURE 1

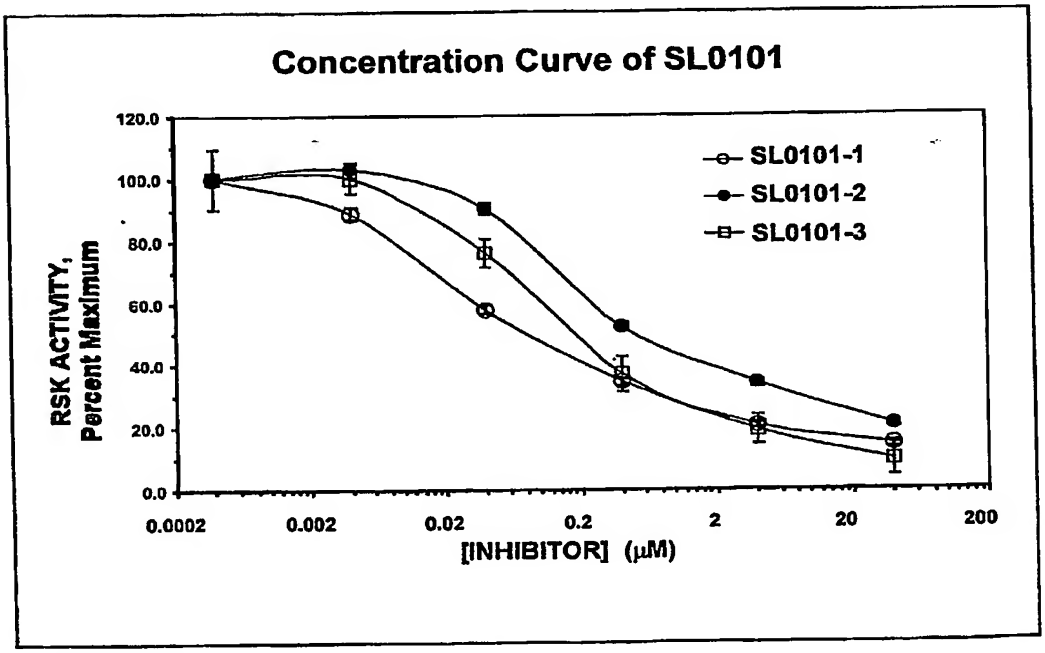


FIGURE 2

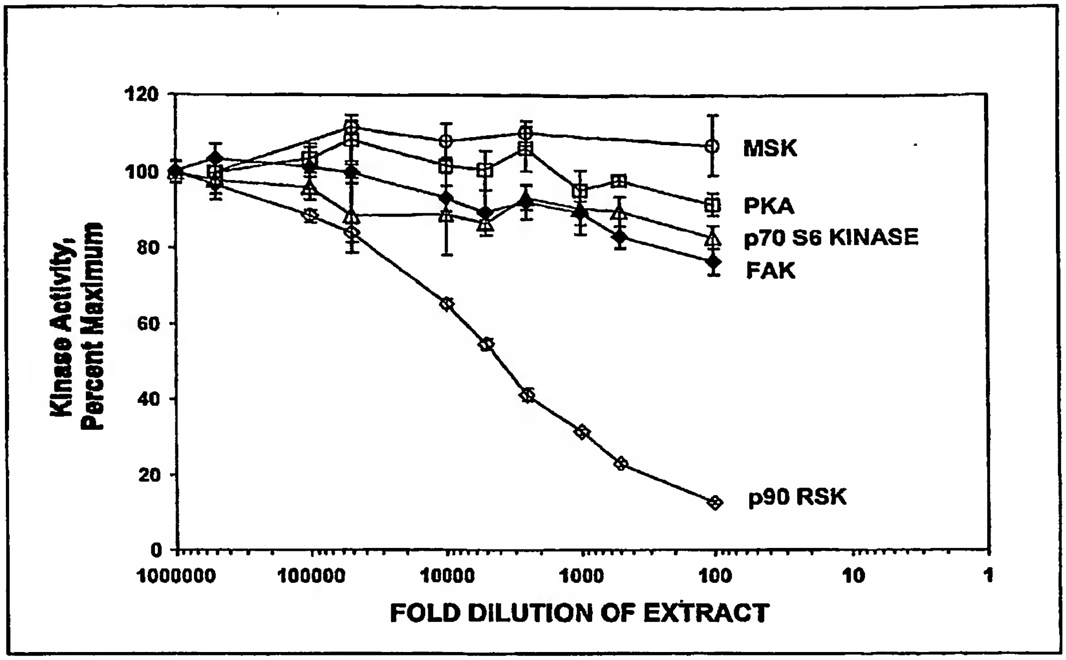


FIGURE 3

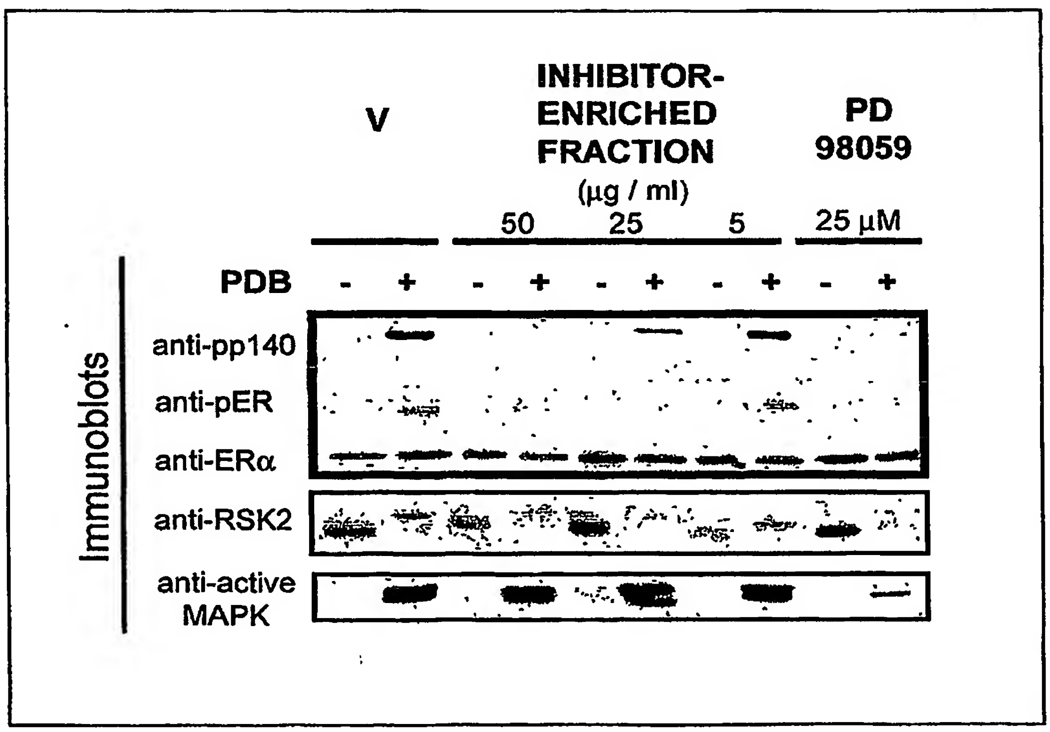


FIGURE 4

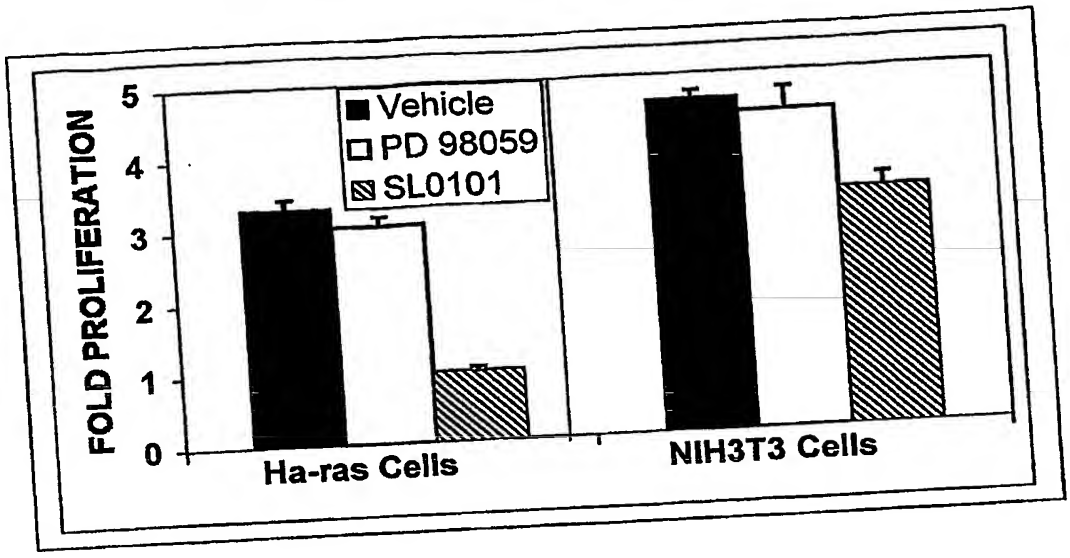

FOLD PROLIFERATION
5
4
3
2
1
0
Vehicle
PD 98059
SL0101
Ha-ras Cells
NIH3T3 Cells

FIGURE 5